Circadian Mechanisms of Prefrontal Cortex Dependent Memory in Trace Fear Conditioning

By

Sage Marie Dobby

Department of Psychology and Neuroscience

University of Colorado Boulder

Boulder, CO 80309

Defense Date: April 11, 2023

Honors Thesis Advisor: Dr. Robert Spencer

Defense Committee:

Dr. Robert Spencer, Thesis Advisor, Department of Psychology and Neuroscience

Dr. Lauren Chun, Honors Council Representative, Department of Psychology and Neuroscience

Dr. Jihye Park, Outside Reader, Department of Chemistry

Table of Contents

1. Abstract 3
2. Introduction 4
3. Materials and Methods 13
4. Results 21
5. Discussion 26
6. Acknowledgements 32
7. References 33

1. Abstract

Circadian rhythms are physiological and behavioral processes that follow an approximately 24-h cycle. They arise from internal biological clocks, which are synchronized mainly by environmental light. Fear-based mental disorders, such as post-traumatic stress disorder (PTSD), are associated with disruptions in circadian rhythms. Understanding this relationship is important for developing more effective treatments. In delayed fear conditioning, the most common form of fear conditioning, the presentation of a tone (conditioned stimulus) is paired with a foot shock (unconditioned stimulus), causing experimental subjects to associate tone presentation with shock delivery. In trace fear conditioning, a brief temporal gap occurs between the tone and foot shock, causing different brain circuits to get engaged. Past work in our laboratory and others has shown circadian rhythms in the extinction of delayed conditioned fear. More recent work in our laboratory has shown that the recall of trace conditioned fear similarly exhibits a rhythm, with recall being stronger during the inactive phase of the rest-activity cycle. The purpose of this experiment was to test whether stronger recall for trace fear is dependent on the time of conditioning or the time of recall. Additionally, I analyzed brain tissue to assess whether rhythms in behavior were reflected in circadian and activity-dependent gene expression in the prefrontal cortex. Rats were fear conditioned at either ZT4 (the inactive phase) or ZT16 (the active phase) and then underwent fear recall after 24 h (same time of day as training), 36 h (opposite time of day as training), and 48 h (same time of day as training). Results showed an interaction between the time of training and the interval between training and testing for trace fear conditioning. Specifically, ZT4 rats showed strong fear recall at all intervals, whereas ZT16 rats showed increased recall over a greater recall interval. The mRNA expression of *Per1*, a key circadian gene, showed opposite results, with the ZT4 expression decreasing over recall intervals

and ZT16 expression remaining constant. For *c-Fos*, an immediate early gene whose expression is used to approximate recent neuronal activation, mRNA expression tended to be higher at ZT16 regardless of the time of training. Notably, however, ZT4-36 h rats showed higher *c-Fos* expression than ZT16-36 h rats in the infralimbic but not prelimbic prefrontal cortex, suggesting differential engagement of the prelimbic cortex during trace fear recall. These results suggest that learned fear may be consolidated more quickly during the inactive phase, leading to signs of prefrontal engagement at later recall intervals during the active phase. In turn, these findings pave the way for circadian assessments, treatments, and prevention for fear-based mental disorders in clinical settings.

2. Introduction

Fear-related mental disorders pose a significant global public health issue. These disorders include anxiety disorders, obsessive-compulsive disorder, and stressor- and trauma-related disorders, such as post-traumatic stress disorder (PTSD) (American Psychiatric Association, 2013). Together, these disorders affect 32% of all individuals in the United States (*National Institute of Mental Health*, 2022). Of people seeking treatment, however, 20–50% do not experience symptom relief (Rosellini et al., 2018; Davidson, J. R. T., et al., 2014).

Fear-based mental disorders appear to involve disruptions in memory processes. For example, patients with PTSD recall fear memories more strongly than controls (Orr et al., 2000, Norrholm et al., 2011). People with PTSD also exhibit memory impairment while learning new memories that help to overcome an acquired fear (Milad et al., 2009). Relatedly, safety memories are recalled poorly in people with PTSD (Acheson et al., 2015, Norrholm et al., 2011). For these

reasons, a detailed understanding of memory mechanisms in fear-based mental disorders may suggest new strategies for risk assessment, treatment, and prevention.

Disrupted circadian rhythms have been found in people who suffer from fear-based mental disorders. After trauma exposure, circadian disruption is a fundamental trait of trauma-related disorders (Agorastos, Olff, 2020). Additionally, many studies have shown that circadian rhythms are a key regulator of memory processes (Hartsock et al., 2022, Krishnan and Lyons, 2015, Rawashdeh et al., 2018). Relatedly, patients with fear-based mental disorders often exhibit disruptions in markers of circadian function, such as alterations in glucocorticoid release patterns (Dayan et al., 2016). Taken together, these pieces of evidence suggest that it may be useful to consider circadian rhythms in memory function as a factor in fear-based mental disorders.

The Circadian System

Circadian rhythms are biological cycles that regulate change throughout the body over a 24-h time period. For example, physiological processes such as hormone secretion, heart rate, energy homeostasis, metabolism, memory, sleep, and blood pressure all exhibit circadian rhythms (Bollinger and Schibler 2014). This internal timing system continues to "free-run" in constant environmental circumstances, such as no fluctuations in constant darkness or temperature (Saini et al., 2015). The circadian system also makes it possible for all bodily tissues to coordinate their activity across the day. Circadian control is crucial for allowing various bodily systems to prepare for daily environmental challenges (Patke, Young, and Axelrod 2020). Conditions like jet lag or working a night shift disrupt circadian rhythms, causing impaired function of tissues throughout the body (Boivin and Boudreau 2014). In fact, shift work

disruption of circadian rhythms can lead to diseases including diabetes, cancer, and cardiovascular disease (Knutsson, 2003).

Circadian rhythms are generated by cellular timekeepers called molecular clocks (Cox and Takahashi 2019). These molecular clocks are found in virtually all of the body's cells, controlling cellular activities in a circadian manner. The molecular clock is a genetic mechanism made up of so-called clock genes and the proteins that result from them (Figure 1). There are four core clock genes: brain and muscle arnt-like protein 1 (*Bmal1),* circadian locomotor output cycles kaput (*Clock),* cryptochrome (*Cry1, Cry2),* and period (*Per1, Per2, Per3)*. BMAL1 and CLOCK proteins first bind to each other and then to DNA E-box sequences found in the promoters of the *Per* and Cry genes to initiate transcription of the *Per* and *Cry* mRNAs. These mRNAs are translated into proteins in the cytoplasm, and a multiprotein PER:CRY complex translocates back into the cell nucleus. The PER and CRY proteins then inhibit the action of the BMAL1:CLOCK heterodimer, suppressing the transcription of the *Cry* and *Per* genes. Once *Per* and Cry levels drop through natural degradation processes, CLOCK/BMAL1 are free to bind again to the *Per* and *Cry* promoters and restart the process. One cycle of the molecular clock takes about 24 hs to complete. In turn, molecular clock products interact with other cellular machinery to generate circadian rhythms in cellular function.

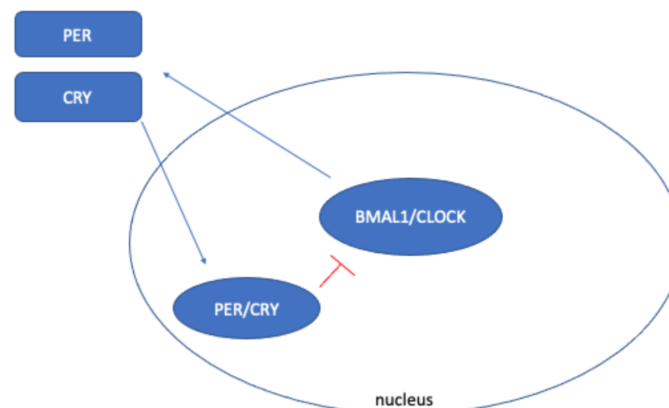

Fig 1. Core clock genes transcription and translation throughout the 24 hour cycle in each cell.

Molecular clocks throughout the body are regulated by a brain region called the suprachiasmatic nucleus of the hypothalamus (SCN). Periodic environmental light entrains, or aligns, the expression patterns of clock genes in the SCN with the solar day (Reppert and Weaver, 2001). Any external or environmental cue that entrains an organism's biological rhythms is known as a zeitgeber, a German word meaning “time giver.” Light is the primary zeitgeber for the SCN and circadian rhythms more generally (Pittendrigh and Minis 1964; Daan and Pittendrigh 1976a; 1976b; Pittendrigh and Daan 1976a; 1976b; 1976c). The SCN receives information about environmental light conditions from ganglion cells in the retinohypothalamic tract (Figure 2) that are innately sensitive to blue light (Güler et al., 2008, Hattar et al., 2002). The protein melanopsin, which retinal ganglion cells use to directly sense light, allows the SCN to detect light without the use of rods or cones (Güler et al., 2008, Hattar et al., 2002). In the SCN, light information is transformed into chemical signals that reset the SCN's clock gene expression cycles through the induction of *Per* gene expression (Reppert & Weaver 2001). This process keeps the SCN clock on a 24-h cycle that is synchronized with the time of day in the surrounding environment.

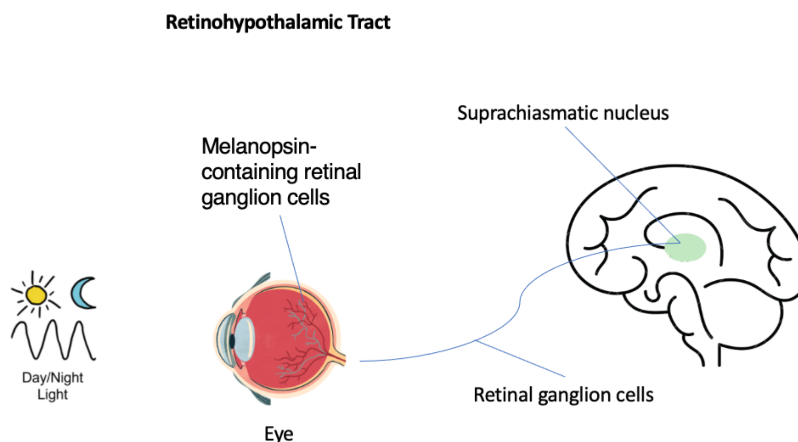

Fig 2. The pathway of the Retinohypothalamic Tract to the SCN

The SCN is the master clock or circadian pacemaker for circadian rhythms in mammals. In rodents receiving a donor SCN transplant, the recipient's biological rhythms shifted to match the new master clock (Ralph et al., 1990). Additionally, in studies where the SCN is lesioned, circadian rhythms throughout the body are disrupted (Saper, 2013). Unexpected exposure to light at night can advance or delay the timing of a person's endogenous clock (Evans & Davidson, 2013). For these reasons, the SCN is considered the master clock for circadian rhythms in mammals.

The SCN uses a bunch of different signals to directly send daily timing information to molecular clocks in other parts of the body. For example, the hypothalamic-pituitary-adrenal (HPA) axis is an important neuroendocrine circuit that controls glucocorticoid release. In the HPA axis, direct signals from the SCN are received by the paraventricular nucleus of the hypothalamus (PVN), which transmits the signals to the pituitary gland (Jones et al., 2021). The pituitary gland, in turn, regulates glucocorticoid release by the adrenal gland. Thus, the SCN generates circadian rhythms in glucocorticoid release (Moore and Eichler, 1972). Glucocorticoids are thought to relay SCN timing signals to molecular clocks in tissues outside the SCN (Spencer et al., 2018). Glucocorticoid exposure in tissues outside the SCN (which, itself, lacks glucocorticoid receptors) drives the expression of the *Per1* gene, acting as a mechanism to reset molecular clock timing (So et al., 2009; Conway-Campbell et al., 2010; Mifsud and Reul, 2016). This occurs through a *Per1* glucocorticoid response element (GRE) in the *Per1* gene promoter, where cortisol receptors can bind. Notably, however, glucocorticoid release is also regulated by stress, suggesting a mechanism by which stress may disrupt

molecular clock function and cellular circadian rhythms (Chun et al., 2018; Mifsud and Reul, 2016; Spencer et al., 2018).

Molecular clocks in cells outside the SCN work together to form tissue-level clocks, called local clocks. Similar to the master clock, local clocks oscillate on a 24-h timescale (Hastings et al., 2003). However, local clocks throughout the body continued to function but were out of sync in a study with lesions to the SCN in rodents, indicating that local clocks are self-sustaining but ultimately controlled by the master clock in the SCN (Yoo et al., 2004). These local clocks can be found in nearly every tissue in the body. These local clocks are important in various brain regions, such as the amygdala, hippocampus, and prefrontal cortex, where they generate circadian rhythms in neuroplasticity and memory-related behavior (Hartsock et al., 2022, Krishnan and Lyons 2015, Rawashdeh et al., 2018). In our work, we are particularly interested in understanding local clocks in relation to learning and memory.

Fear-Related Memory and the Prefrontal Cortex

A common paradigm for examining the biological foundations of memory is Pavlovian fear conditioning. This is referred to as Pavlovian fear conditioning because of Ivan P. Pavlov's psychological concept of classical conditioning (Pavlov, 1927). Classical conditioning is the practice of training a subject to respond to an unconditioned stimulus by using a conditioned stimulus (i.e. a dog salivates when a bell is rung, thinking it will receive food even when food is not present) (Rehman et al., 2022). In the most common behavioral procedure for fear memory, called delayed fear conditioning, a rat is placed in a behavior box, or context, and presented with a neutral cue (the conditioned stimulus, often a tone) that is paired with an innately aversive stimulus (the unconditioned stimulus, often a foot shock) (Davis, 1992; LeDoux, 2000; Maren,

2001). Notably, in delayed fear conditioning, the onset of the tone precedes the delivery of the shock, but the tone is still present when the shock is given. Through pairings of the tone with the shock, the rat learns that the tone predicts the shock. When in danger, rats exhibit defensive behaviors such as freezing, much like a deer in headlights. Shock can automatically cause freezing behavior (unconditioned response), while tone can do the same only after being paired with the shock (conditioned response).

If the same rat is placed in a different context, it will freeze again if the tone is played. Freezing in response to the tone is often used as a measure of fear memory recall. After the tone is played multiple times by itself, however, the rat develops a new memory that the tone is no longer anything to be afraid of, at least in this new context. At this point, the fear response is extinguished, and we measure this through reductions in freezing behavior (Bouton, 2000, 2002; Hermans et al., 2006; Maren et al., 2013; Vervliet et al., 2013; Goode and Maren, 2014). Rather than erasing the preexisting fear memory, the brain engages new pathways that learn to no longer fear the stimulus (Maren and Chang, 2006; Myers et al., 2007; Maren, 2014). For these reasons, we call this learning process conditioned fear extinction, and the resulting memory is called an extinction memory, which can be tested in a later session by presentation of just the tone again.

There are many brain regions involved in delayed conditioned fear and extinction, most notably the amygdala, hippocampus, and prefrontal cortex (Orsini & Maren, 2012, Giustino & Maren, 2015). The amygdala supports fear learning and recall (Nitecka and Ben-Ari, 1987; McDonald and Augustine, 1993; Paré and Smith, 1993; Royer et al., 1999; Lee et al., 2013; Duvarci and Pare, 2014). The hippocampus plays a significant part in the fear circuit, given its function in encoding spatial and contextual information (Selden et al., 1991; Kim and Fanselow, 1992; Phillips and Ledoux, 1992). There are two main divisions of the prefrontal cortex in rats

that play important functions in the fear circuit. The prelimbic cortex (PL) is thought to promote fear memory expression, whereas the infralimbic cortex (IL) is thought to promote fear memory suppression (Quirk and Beer, 2006; Sotres-Bayon and Quirk, 2010; Milad and Quirk, 2012; Riga et al., 2014).

There is another fear memory paradigm called trace conditioned fear. Trace conditioned fear is similar to delayed conditioned fear, but trace conditioned fear has a short time interval between when the tone ends and the shock is delivered. With this one small change in the paradigm, the prefrontal cortex is engaged differently. In the delayed fear conditioning paradigm, the prefrontal cortex (particularly the IL) appears to be of special importance for recalling extinction memories. But in trace fear conditioning, the prefrontal cortex is thought to be of equal importance for recall of conditioned fear memories. For example, prefrontal cortex neurons become activated during the time interval between the tone and the shock (Kwapis et al., 2014). This is thought to help create memories associated with fear expression (Gilmartin et al., 2014). But compared to delayed conditioned fear and extinction, the neuroanatomy of trace conditioned fear and extinction have not yet been well studied.

Circadian Rhythms in Fear Memory

Our laboratory has characterized clock gene expression cycles in the prefrontal cortex, revealing that the prefrontal cortex contains a robust local clock (Chun et. al, 2015). As noted above, the part of the delayed fear paradigm that depends most on the prefrontal cortex is extinction recall (Gonzalez & Fanselow 2020). Extinction recall exhibits a circadian rhythm in rodents and humans, and extinction recall tends to be best during the active phase (Chaudhury et al., 2002, Woodruff et. al, 2015, Woodruff et al., 2018, Pace-Schott et al., 2013, Hartsock et al.,

2022). Importantly, this rhythm is abolished by disrupting clock gene expression in the prefrontal cortex (Woodruff et al., 2018). These findings suggest that a local clock in the prefrontal cortex contributes to circadian rhythms in delayed conditioned fear extinction. For this reason, our laboratory is investigating whether other prefrontal cortex-controlled brain processes may also display circadian rhythms.

Recent work in our laboratory has shown that rats exhibit a time-of-day difference in both trace fear learning and trace fear recall. Rats learning trace fear during their inactive phase show a larger percentage of time spent freezing (unpublished observations, Fausnaught, 2021). Similarly, rats freeze more when undergoing trace fear recall during the inactive phase (unpublished observations, Fausnaught, 2021). This raises the question of whether time-of-day differences in trace fear recall depend on the time of day of learning or the time of day of recall. In order to find out, our laboratory has previously carried out another experiment in which rats were trace fear conditioned in either the inactive or active phase. Trace fear was then recalled by these rats 24 hs, 36 hs, or 48 hs following the training session. Rats trained during the inactive phase exhibited higher freezing 24 hs after learning compared to rats trained during the active phase; however, 36 and 48 hs after learning, there was no discernible difference in percent freezing. To investigate this finding more deeply, I sought to understand prefrontal cortex mechanisms underlying these behavioral responses.

Immediate early genes are genes whose transcription is activated by neural activity. For example, *c-Fos* is an immediate early gene with expression that correlates with neuronal activity (Kovács, 2008). Upon neuronal stimulation, multiple intracellular pathways converge upon response elements in the *c-Fos* promoter. In the absence of stimulation, however, *c-Fos* rapidly decays. For this reason, *c-Fos* is often used as a correlate of neuronal activation. Interestingly,

the clock gene *Per1* is often expressed in the prefrontal cortex in patterns similar to *c-Fos* (Chun et al., 2018), potentially because of similar response elements that are found in the promoter of the *Per1* gene (Yamamoto et al., 2005).

For these reasons, I hypothesized that prefrontal cortex *Per1* and *c-Fos* levels might vary with freezing behavior during trace fear recall, with *Per1* and *c-Fos* mRNA levels being the highest for rats trained at ZT4. I think this because higher freezing behavior might require increased neuronal activity to support it. In my project, I examined brain tissue from rats in the experiment described above. I specifically measured *c-Fos* and *Per1* expression in the prefrontal cortex after trace conditioned fear recall occuring 24, 36, and 48 hs after trace fear conditioning. I found that the story was more complex, with neither *c-Fos* nor *Per1* mRNA expression patterns mirroring freezing. Instead, my data showed signs of prefrontal engagement at later recall intervals during the active phase.

3. Materials and Methods

The behavioral experiment and all rodent work were performed by a previous Honors student in the Spencer Laboratory (Fausnaught, 2021). Here, I provide details of the experiment for the sake of completeness. My work for this thesis project began with the slicing of brain tissue, and I have also completed additional behavioral analyses to present alongside my mechanistic brain measurement findings.

3.1 Animals

The rats used in this experiment were adult male Sprague-Dawley rats from Envigo Laboratories (Indianapolis, IN). They were housed in pairs in polycarbonate tubs measuring 47

cm x 23 cm x 20 cm (Allentown Caging Equipment Company, Allentown, NJ). All of the rats were provided unlimited access to food (Tekland Rodent Diet 8640; Harlan) and water during all tests. After arriving, the rats were housed in separate rooms and acclimated to the proper 12:12 h light:dark cycles. All of the behavioral tests were performed at either zeitgeber time (ZT) ZT4 (four hs after light onset) or ZT16 (four hs after light offset). The ZT4 tests were performed under white light, and the ZT16 tests were performed under dim red light. All of the rats were transferred from their housing room to the testing room in their home cages, which were wrapped in black plastic bags (Hefty 55g bags; Reynolds Consumer Products, Inc.; Lake Forest, IL) to block out light and keep transportation conditions constant. All procedures were carried out in compliance with the ethical treatment of animals under the approval of the Institutional Animal Care and Use Committee at the University of Colorado.

3.2 Experimental Procedure

The purpose of this experiment was to determine if rats who underwent trace fear conditioning had better recall depending on the time of learning or the time of recall. To test this, 36 rats were split into 6 different treatment groups (n=6, N=36). Rats were trace fear conditioned at either ZT4 or ZT16 and then tested for trace fear recall at 24, 36, or 48 hs. Thus, the six different treatment groups for this experiment were as follows 1) rats conditioned at ZT4 and tested 24 hs later at ZT4, 2) rats conditioned at ZT4 and tested 36 hs later at ZT16, 3) rats conditioned at ZT4 and tested 48 hs later at ZT4, 4) rats conditioned at ZT16 and tested 24 hs later at ZT16, 5) rats conditioned at ZT16 and tested 36 hs later at ZT4, and 6) rats conditioned at ZT16 and tested 48 hs later at ZT16. The 24-h and 48-h groups recalled trace fear during the same phase in which they were trained, whereas the 36-h group recalled trace fear in the opposite

phase. The experiment was designed this way to test if time-of-day differences in the recall of trace conditioned fear depend on the time of day of learning (reflecting rhythms in memory consolidation processes) or the time of day of recall (reflecting rhythms in memory retrieval processes). Additionally, the 48-h groups were added to make sure that any differences between rats tested after 36 h and those tested after 24 h were likely caused by the time of day the rats were assessed rather than the amount of time that passed between training and testing.

Trace fear conditioning took place in Context A. Context A was a rectangular box with two stainless steel walls and two plexiglass walls (St Albans, VT). A current generator (Med Associates Inc.; ENV-414S; St Albans, VT) and speakers (Med Associates Inc.; ENV-025F; St Albans, VT) were mounted on top of the boxes to create the tone, and the floor was made of stainless steel bars. Percent time spent freezing, rather than being recorded manually, was scored through automation (VideoFreeze Software version 3.0.0.0, Med Associates Inc.; SOF-843). To establish a baseline for freezing behavior, the rats were kept in Context A for five minutes before tone-shock pairings occurred. The speakers played a 15-second tone (3kHz at 80dB) following the baseline period. For ten seconds after the tone, no tone was played, and no shock was delivered. Following those ten seconds, a 1-second, 0.8mA foot-shock was delivered to complete the trial. The ten seconds between the tone and shock is referred to as the “trace” period because, after the first tone-shock pairing, rats must remember a memory “trace” of the tone-shock association during this time. All experimental groups underwent 6 trials in total, each separated by an intertrial interval of 240 seconds.

The rats were placed in a different context, Context B, for conditioned fear recall either 24, 36, or 48 hs later. Context B was the same box as in Context A, but with a curved plexiglass wall lining the interior and a smooth plexiglass floor covering the shock grid. The box was

scented with peppermint oil. Three minutes of baseline data were collected during this fear recall session. Then 3 tones were played, each followed by a ten-second trace interval and a 240-second intertrial interval. No shock was delivered. Here, only three trials were given, since fear recall was designed to measure memory of conditioned fear from the previous session (and not to induce extinction memory, as described in the introductory section of this thesis).

3.3 Tissue Preparation

Following trace fear recall, rats were sacrificed using rapid decapitation. The brains were immediately removed, frozen over dry ice, and then stored at a temperature of -70°C in an ultra-low freezer. Using a CM1850 cryostat (Leica, Buffalo Grove, IL), brain sections were coronally cut at a thickness of 12 μm. Comparisons with a Rat Brain Atlas were used to determine the proper orientation of the brain sections and the structural landmarks for the prefrontal cortex collection (Paxinos and Watson, 2013). The brain slices were collected using a standard thaw-mounting technique on Superfrost Plus glass slides (Fisher Scientific, Hampton, NH) and stored at -70°C.

3.4 *In Situ* Hybridization

These slides were then put through a radioactive *in situ* hybridization procedure to gather quantitative *Per1* mRNA levels in the prefrontal cortex. The following steps were followed from a protocol previously used in the lab (Girotti, Weinberg, & Spencer, 2009). This procedure uses a single-stranded mRNA probe that is attached to a radioactive isotope, S35, to label the target mRNA. In our case, we used a single-stranded *Per1* DNA template to build a radioactive RNA probe against *Per1* mRNA, which we then used to label *Per1* mRNA in our prefrontal cortex

tissue. This enabled us to quantify *Per1* mRNA expression in the prefrontal cortex following trace fear recall. After the slides were gradually dehydrated with increasing concentrations of ethanol, the probe was reverse pipetted onto coverslips, to which the slides were applied. The complementary radioactive probe could then bind specifically to the *Per1* mRNA in the tissue. The slides were left to incubate at 54°C overnight to promote hybridization. After this overnight incubation, the slides were put through a 1 h wash at 37°C that used an RNase digestive enzyme to degrade any excess unbound probe. After multiple washes in saline sodium citrate and an incubation at 65°C to degrade the RNase, the slides were again dehydrated in increasing concentrations of ethanol and exposed to a Biomax MR Film (Carestream Health, Rochester, NY) in light-proof FBXC autoradiography cassettes (Fisher Scientific, Hampton, NH). The X-ray film detects signals from the radioactive labeled mRNA, which provides quantitative data regarding the expression and location of *Per1* mRNA in the tissue.

After five weeks, the films were developed using a SRX-101A Mini-Medical Film Developer (Konica Minolta Medical & Graphic Incorporated, Wayne, JN). The films were imaged through the use of a Northern Light B96 lightbox (Imaging Resources Incorporated, Albany, OR) and a XS-ST70 CCD camera (Sony, New York City, NY) with a 7000 zoom lens (Navitar, Rochester, NY). The pictures were converted to digital format and saved using Scion Image software version ß4.0.2 (Scion Corporation, Frederick, MD).

We measured the four darkest sections from each set of prefrontal cortex slides. Densitometry analysis was then performed, and the analyst was blinded to the treatment groups. We used the Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 2007) to determine the boundaries of various parts of the prefrontal cortex: the infralimbic prefrontal cortex (IL), cingulate cortex (CG), ventral orbital prefrontal cortex (VO), and prelimbic prefrontal cortex

(PL). We then used ImageJ software version 4 (National Institutes of Health, Bethesda, MD) to measure the optical density in each of our regions of interest. Using Excel, the optical density means and standard errors for all of the treatment groups were calculated, and additional analyses were run in Prism version 9.1.1 (GraphPad Software, San Diego, CA).

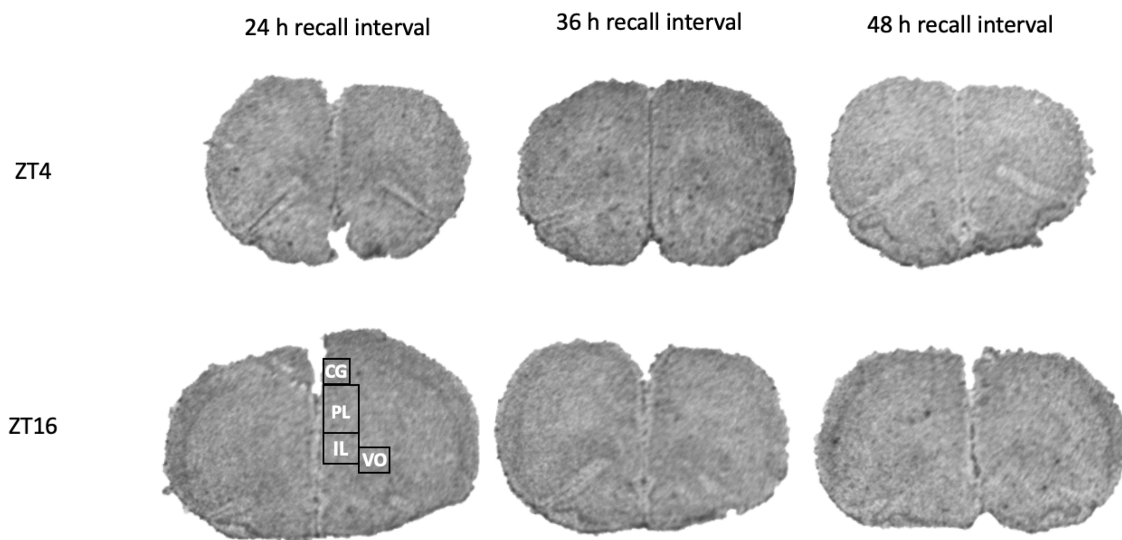

Fig 3. Radioactive *in situ* hybridization film images from the PFC

To analyze the mRNA levels of **both** *Per1* and *c-Fos*, additional thaw-mounted slides were run through a different process called fluorescent *in situ* hybridization (FISH). FISH is a technique used to visualize specific RNA or DNA sequences within a cell or tissue sample. FISH is useful for estimating the qualitative amount of expression of *Per1* and *c-Fos* mRNA, enabling us to count cells in which *Per1* and/or *c-Fos* mRNA were present. *Per1* and *c-Fos* were both being labeled in this procedure; hence, it is referred to as a double-labeled FISH.

The double-labeled FISH used for this experiment followed a defined protocol that is referenced for the following section. This protocol was used concurrently with the TSA Fluorescence Systems Kit from NEN Life Sciences (Fremont,CA). Day one of FISH began with making mRNA riboprobes for both *Per1* and *c-Fos* using Digoxigenin-UTP and Fluorescein-UTP, respectively. Creating these probes followed the same basic procedure for radioactive *in situ* hybridization. Following, the probes were reverse pipetted onto the coverslips (VWR, Radnor, PA), and the slides were left to incubate at 37°C overnight. The following day, the slides' coverslips were floated off in saline sodium citrate solution and then incubated in RNase for 1 h at 37°C. As above, slides were then washed with saline sodium citrate and incubated for an h at 65°C to degrade the RNase. Afterwards, the slides were cooled to room temperature, rinsed in saline sodium citrate, and transferred to coplin jars containing 0.05 M Phosphate Buffered Saline (PBS) overnight. The next day the slides were prepared with a blocking buffer and each unique (*Per1* or *c-Fos* mRNA) anti-hapten horseradish peroxidase (HRP). An anti-hapten HRP is an HRP-conjugated antibody that binds to the hapten-labeled probe (digoxigenin or fluorescein). HRP is an enzyme used to “tag” the mRNA with the fluorophore-labeled amplification reagent. These steps are important to catalyze the deposition of the fluorophore. Once slides were incubated with both anti-dig and anti-fluorscein HRP conjugated antibodies followed by the incubation with the TSA tyramide reagents, the slides were fluorescently tagged and ready for the final coverslip to be looked at under the microscope. The slides were imaged using a Zeiss AXIO Imager M1 microscope (Wetzlar, Germany) at 10x magnification (Figure 4) . FISH *Per1* data were excluded from further analysis due to high constitutive mRNA labeling that limited the utility of a cell count strategy. Cells positive for

c-Fos mRNA (Figure 5) were then counted using QuPath software version (0.3.2) and analyzed in Excel and Prism.

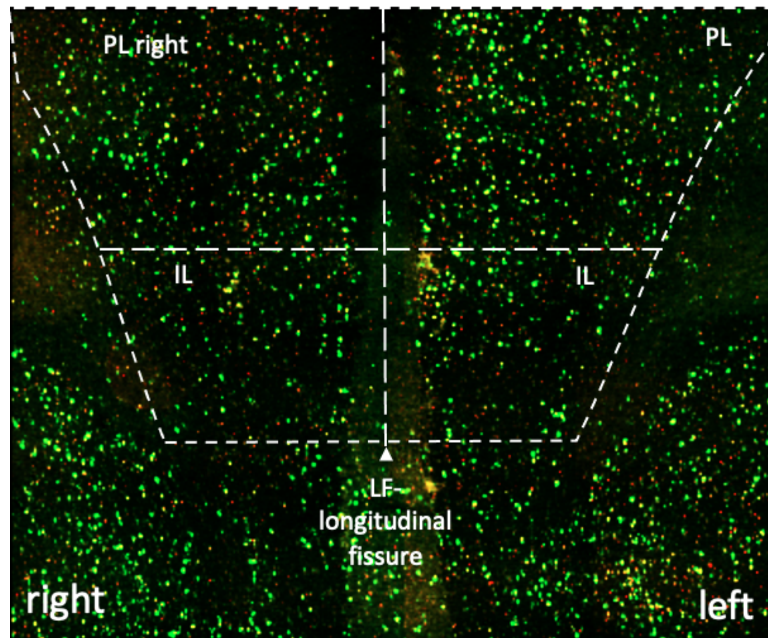

Fig 4. Double-Labeled fluorescent *in situ* hybridization showing both *c-Fos* and *Per1*

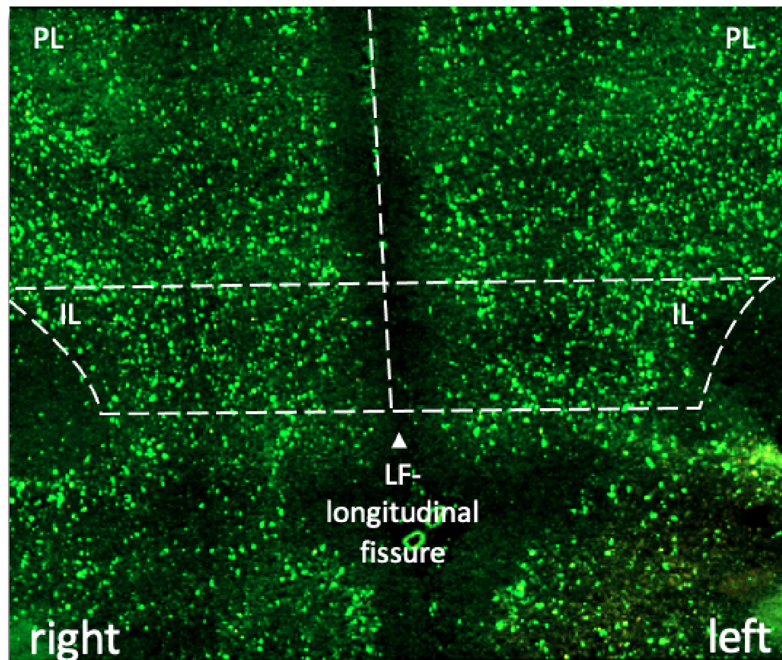

Fig 5. Fluorescent *in situ* hybridization showing c-Fos

3.5 Statistical Analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA), which is a statistical technique used to compare the means of multiple groups. We tested two independent factors, training ZT and fear recall interval, as well as their interaction. We used Fisher's Least Significant Difference post hoc tests for multiple comparisons. Results were considered significant when there was a *p*-value less than 0.05. Graphs depict treatment group means and standard error of the means.

4. Results

4.1 Behavior

To examine freezing behavior between treatment groups, rats were autonomically scored for percent time freezing during trace fear recall. Results showed no main effects or significant interactions between training ZT and recall interval during the baseline period of the recall session (Fig. 6). During tone presentation, rats in all groups froze more, and there was a significant interaction between ZT and recall interval (**$F_{(2,30)} = 5.050, p < 0.05$**), such that rats trained at ZT16 froze less than rats trained at ZT4 during the 24 h fear recall session but showed increased freezing from 24 h to 48 h (Fig. 7). Freezing behavior during the trace period showed a main effect of training ZT (**$F_{(1, 30)} = 10.08, p < 0.01$**), again with ZT16 rats freezing less, especially at the 24 h recall interval (Fig. 8). During the intertrial interval, there was a significant main effect of training ZT (**$F_{(1, 30)} = 11.11, p < 0.01$**), with ZT16 rats freezing less overall (Fig. 9). Notably, however, post hoc tests showed reduced freezing in ZT16 rats tested also at 48 h, which was not seen in any of the other behavioral graphs.

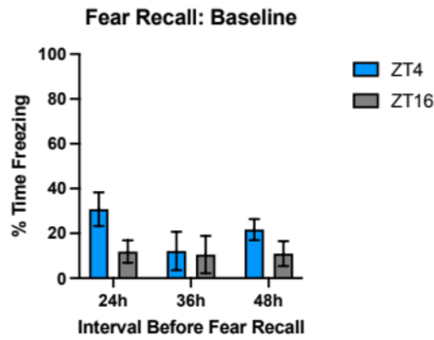

Fig. 6 This graph shows the average percent freezing across all three testing intervals during the baseline period of the fear recall session. Rats in all treatment groups showed low levels of freezing during the baseline portion of the behavioral experiment. There were no main effects or significant interactions.

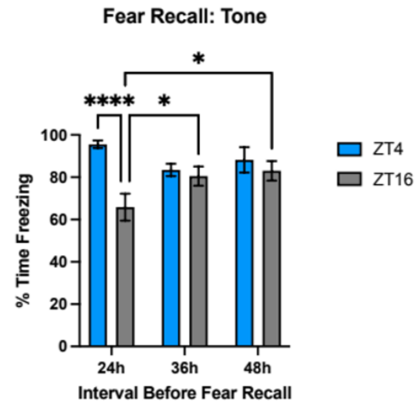

Fig. 7 Rats in all groups froze when the tone came on during trace fear recall. There was a significant interaction between the training ZT and recall interval (**$F_{(2,30)}$ = 5.050, $p < 0.05$**). This was evident in reduced freezing at ZT16 compared to ZT4 at 24 h and an increase in freezing in ZT16 rats across recall intervals.

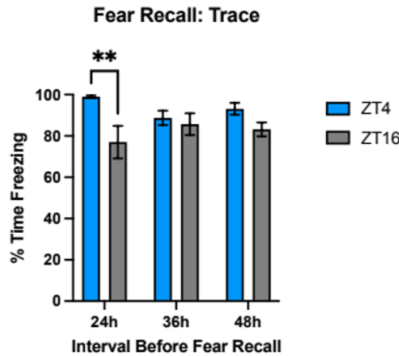

Fig. 8 Rats in all groups continued to freeze during the trace portion of the behavioral experiment. This is the "trace" interval, when the tone stops playing but the shock has not yet been delivered. There is a main effect of ZT (**$F_{(1, 30)}$ = 10.08, $p < 0.01$**), with ZT4 freezing being higher than ZT16 freezing overall. Posthoc test showed that this is driven in part by significantly lower freezing in ZT16 rats at 24 h.

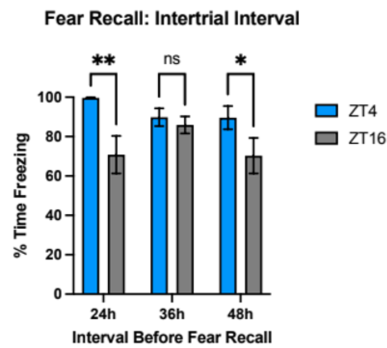

Fig. 9 Rats in all groups continued to freeze in the interval between each trial. There is a main effect of ZT (**$F_{(1, 30)}$ = 11.11, $p < 0.01$**), and posthoc tests showed reduced freezing in ZT16 rats tested at 24 h and 48 h.

4.2 *Per1* mRNA Expression in the Prefrontal Cortex

To assess time-of-day differences in *Per1* mRNA expression in the prefrontal cortex, radioactive *in situ* hybridization was used. There were no significant interactions or main effects in the CG or PL (Fig. 10, Fig. 11). However, there was a significant main effect of recall interval in the IL (**$F_{(2, 30)} = 3.321$, $p < 0.05$**) (Fig. 12) and a significant interaction between training ZT and recall interval in the VO (**$F_{(2, 30)} = 3.321$, $p < 0.05$**). Notably, there were also trending interactions in the CG (**$F_{(2, 30)} = 3.262$, $p = 0.052$**), IL (**$F_{(2, 30)} = 2.809$, $p = 0.076$**), and PL (**$F_{(2, 30)} = 2.968$, $p = 0.067$**), suggesting a similar pattern of *Per1* expression across prefrontal cortex subdivisions. Post hoc tests revealed that *Per1* mRNA expression declined by the 48-h recall interval in ZT4 rats, whereas *Per1* expression remained fairly constant at all recall intervals in ZT16 rats. Thus, at the 48 h test interval, ZT4 rats consistently showed reduced *Per1* expression compared to ZT16 rats, although this effect was significant in post hoc testing only in the VO (Fig. 13).

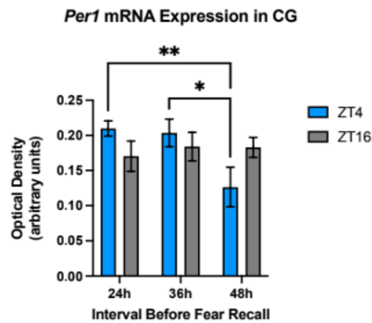

Fig. 10 In the CG region of the PFC, there was a trending interaction between training ZT and recall interval (**$F_{(2, 30)} = 3.262$, $p > 0.05$**). Unprotected posthoc tests showed a significant decrease in *Per1* expression between ZT4 rats tested at 24 h versus 48 h, mirroring a significant decrease in *Per1* expression between ZT4 rats tested at 36 h versus 48 h.

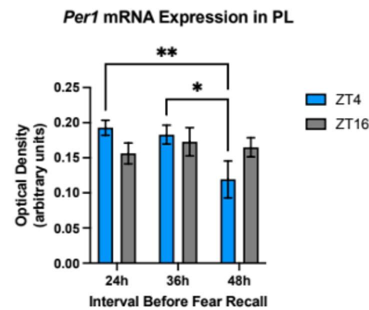

Fig. 11 In the PL PFC brain region, there was a trending interaction between training ZT and recall interval (**$F_{(2, 30)} = 2.968$, $p > 0.05$**). According to unprotected posthoc tests, there is a significant decrease in *Per1* expression between ZT4 rats tested at 24 h versus 48 h, reflecting a significant decrease in *Per1* expression between ZT4 rats tested at 36 h versus 48 h.

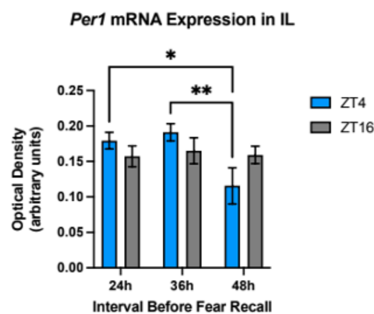

Fig. 12 In the IL PFC brain region, there is a main effect of recall interval (**$F_{(2, 30)} = 3.321$, $p < 0.05$**). Posthoc tests showed that this effect was driven by a significant reduction in *Per1* expression in ZT4 rats from 24 h to 36 h (**$p < 0.01$**), and from 24 to 48 h (**$p < 0.05$**).

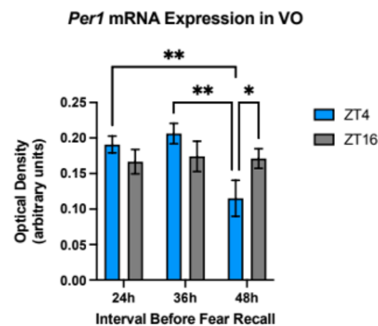

Fig. 13 In the VO region of the PFC, there is a significant interaction between training ZT and recall interval (**$F_{(2, 30)} = 3.704$, $p < 0.05$**). There is a significant reduction in *Per1* expression in ZT4 rats tested at 24 h versus 36 h and 24 h versus 48 h. Additionally, ZT4 rats exhibited reduced *Per1* expression compared to ZT16 rats at 48 h.

4.3 *c-Fos* mRNA Expression in the Prefrontal Cortex

Results from the fluorescent *in situ* hybridization for *c-Fos* mRNA showed time of training day differences as well as some differences between the PL (Fig. 14) and IL (Fig. 15) in the pattern of the interaction between training ZT and recall interval. There was a significant interaction between training ZT and recall interval in both the PL (**$F_{(2, 29)} = 8.914$, $p < 0.01$**) and IL (**$F_{(2, 29)} = 11.18$, $p < 0.001$**). Whereas rats in the ZT4-24 h and ZT4-48 h groups showed similar *c-Fos* mRNA expression, rats in the ZT4-36 h group showed much higher *c-Fos* mRNA expression. Oppositely, rats in the ZT16-24 h and ZT16-48 h groups showed significantly higher *c-Fos* mRNA expression compared to rats in the ZT16-36 h group (although this post hoc result was not significant when comparing ZT16-24 h and ZT16-36 h groups in the PL). Notably, however, in the IL, there **was** a significant difference between ZT4 and ZT16 rats during the 36 h recall interval, whereas there **was not** a significant difference at 36 h in the PL.

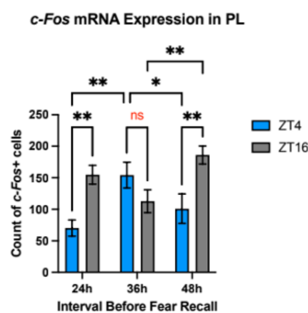

Fig. 14 In the PL region of the PFC, for c-Fos mRNA expression, there is a significant interaction between training ZT and recall interval (**$F_{(2, 29)} = 8.914$, $p < 0.01$**). Posthoc tests revealed significantly greater *c-Fos* expression at ZT16 at 24 h and 48 h. Notably, there is **no** significant difference between ZT4 and ZT16 at 36 h. Rats trained at ZT4 showed greater *c-Fos* expression at 36 h than 24 h or 48 h, whereas rats trained at ZT16 showed lesser *c-Fos* expression at 36 h than 48 h.

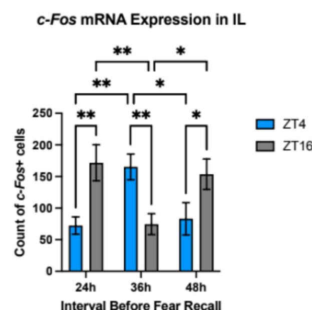

Fig. 15 In the IL region of the PFC, there is a significant interaction between training ZT and recall interval (**$F_{(2, 29)} = 11.18$, $p < 0.001$**). All rats that underwent fear recall at ZT16, including rats that were trained at ZT4, showed increased IL *c-Fos* expression compared to rats that underwent fear recall at ZT4. Posthoc tests revealed testing at ZT16 drove higher *c-Fos* expression than rats tested at ZT4 during the 24 h, 36 h, and 48 h recall interval. These tests showed that the ZT4-24 h and ZT4-48 h groups showed less *c-Fos* expression than the ZT16-24 h and ZT16-48 h groups, whereas the ZT4-36 h group showed more *c-Fos* expression than the ZT16-36 h group. In addition, the ZT 16-36 h group showed less *c-Fos* expression than the ZT16-24 h and ZT16-48 h groups, and the ZT4 rats showed the opposite pattern. There is a significant difference between every ZT training its corresponding recall interval.

5. Discussion

My hypothesis for this experiment was that time-of-day differences in freezing behavior would be mirrored by *Per1* and *c-Fos* mRNA expression in the prefrontal cortex, particularly in the IL and PL, which are critical for fear-related learning and memory in delayed fear conditioning. Our results did not support this hypothesis for either gene. In both cases, we saw interactions between training ZT and recall interval, and these patterns differed from behavior and from each other.

Trace fear conditioning is not well understood, so all data from this experiment are important for understanding the process and underlying brain circuitry. Prior to this project, circadian rhythms in fear-related memory and mechanisms were only understood for delayed fear conditioning (Chaudhury and Cowell, 2002; Pace-Schott et al., 2013; Woodruff et al., 2015; Woodruff et al, 2018; Hartsock and Spencer, 2022). Here, we found a clear time-of-day difference for fear recall in the trace fear paradigm. The baseline behavior does not support a time-of-day difference in baseline fear, which suggests that any time-of-day differences have to do with the tone-shock association. The tone, trace, and intertrial interval all show significant time-of-day differences. During the tone, ZT4 rats seem to be freezing more than ZT16 rats at the 24 h recall interval, but over time this effect seems to go away, with no time-of-day differences being seen at 36 and 48 h. For both the tone and trace intervals, ZT4 freezing started high and remained high across all recall intervals, whereas ZT16 freezing started low and increased across recall intervals. During the intertrial interval, there was a different pattern compared to the tone and trace intervals, that being that ZT16 rats exhibited low freezing at not only 24 h but also 48 h. Notably, this does not happen for ZT4-36 h rats. These findings suggest two things. First, trace fear recall <u>during the tone and trace</u> depends on both the time of training

and the recall interval. Second, trace fear recall during the intertrial interval depends on the time of learning and the time of recall.

To explain the first finding, consider that the ZT16 trace fear memory gets stronger over time, consistent with the possibility that the fear memory may take longer to consolidate if learned at ZT16. During a rat's inactive phase, trace fear conditioning may have more of an effect because the rat is less alert and active. The initial shock may be more startling for a ZT4 than ZT16 rat. Whereas the ZT4 rat should be sleeping, the ZT16 rat is more awake and, likely, coherent. In general, this may explain why fear learning is better in the inactive phase during the tone and trace. Another possibility that explains why ZT16 rats freeze less could be that all the activity occurring within a rat's active phase competes with freezing behavior, causing rats to freeze less. To explain the second finding, we suggest that ZT16 rats are better able than ZT4 rats to distinguish the difference between the tone and trace compared to the intertrial interval. During the tone and trace, rats at both ZT4 and ZT16 should assume the shock is coming. In other words, they should recognize the tone and trace as predictive of danger. On the other hand, the intertrial interval should be recognized as a period of safety, with shock not being imminent. It appears that, regardless of the time of training, rats cannot distinguish the intertrial interval as safe if tested at ZT4. But, rats trained at ZT16 (and not ZT4) can still distinguish the intertrial interval as safe when tested at ZT16, as seen by less freezing in ZT16 rats at the 24 h and 48 h recall intervals.

The purpose of measuring *Per1* mRNA levels in the prefrontal cortex was to see if there was a time-of-day difference in trace fear recall-induced *Per1* at different recall intervals. *Per1* mRNA basal transcription is regulated by BMAL1 until it reaches a peak level that then through PER1 feedback inhibition of BMAL1 inhibits *Per1* transcription. This peak occurs in prefrontal

cortex local clocks during the early active phase (Chun et al., 2018), which in rats includes ZT16. Although we had a significant interaction between training ZT and recall interval only in the VO, a similar trend was present for *Per1* mRNA expression across all prefrontal cortex regions. There was high *Per1* expression in ZT4-24 h and ZT4-36 h rats, but ZT4-48 h rats showed a decrease in *Per1* mRNA expression compared to the other ZT4 recall intervals. On the contrary, ZT16 rats showed high *Per1* mRNA expression in all brain regions across all recall intervals. Normally, *Per1* mRNA expression in the prefrontal cortex would be lowest around ZT4 and highest around ZT16. Our data show this not to be true after trace fear recall at 24 h (when ZT4 *Per1* was high during testing at ZT4) and 36 h (when ZT16 *Per1* was high during testing at ZT4). Given that *Per1* acts like an immediate early gene in the prefrontal cortex (Chun et al., 2018), we expected that fear recall might induce *Per1* mRNA expression. It is possible that prefrontal activation occurs only during the consolidation phase of the memory process. Thus, the ZT4-24 h rats and ZT16-36 h rats might have had heightened *Per1* mRNA expression due to the memory still being consolidated. Relatedly, the decrease in *Per1* mRNA expression for ZT4-48 h rats could be due to the fear memory being as consolidated as needed for fear learning, and therefore no need to drive prefrontal cortex immediate early gene expression for ZT4-48 h rats because the memory is done consolidating, showing a decrease in *Per1* mRNA expression. Our data cannot say whether ZT16 *Per1* mRNA expression would continue to be high after the 48 h recall interval, since we did not test later recall intervals. Additionally, the ZT16 trace fear memory appears to be consolidated by the 48 h recall interval. Therefore, our data cannot tell us much about differences in *Per1* induction between our ZT4 and ZT16 groups. All we can conclude is that *Per1* expression was elevated above likely basal levels (as evidenced by low *Per1* in ZT4 rats at 48 h)

after our ZT4 rats underwent trace fear recall at 24 h and after our ZT16 rats underwent trace fear recall at 36 h.

The results from the *c-Fos* mRNA cell counts show that the PL and IL both had significant interactions between training ZT and recall interval. Notably, this was because *c-Fos* mRNA expression was highest in rats tested at ZT16 regardless of the time of training. However, in the IL, there was a significant difference in *c-Fos* mRNA expression between ZT4 and ZT16 during the 36-h recall interval, whereas in the PL there was not a significant difference. Previous work has shown circadian rhythms in *c-Fos* expression in the brain (Cirelli et al., 2004; Baltazar et al., 2013). Our results seem to support that this rhythm is present in the rat prefrontal cortex, given the clear time-of-day difference in *c-Fos* expression in the IL, regardless of training ZT. Given that IL neurons are thought to be involved in the recall of delayed fear extinction, it is plausible that, because the rats in our experiment never learned to extinguish fear, presentations of the tone never activated the IL. On the other hand, the PL is known to be activated during delayed fear recall. Our results suggest that trace fear recall can also activate the PL, but only above basal levels in rats trained at ZT16 but tested at ZT4 (ZT16-36 h group). As the majority of prefrontal cortex neurons are excitatory, the glutamate pyramidal neurons that project to the amygdala are probably the source of the abundance of *c-Fos* in prefrontal cortex regions. Our *c-Fos* mRNA results suggest that some sort of prefrontal cortex-dependent emotional learning circuitry could be involved specifically within the PL during trace fear recall.

The behavior results indicate a consistently higher percent freezing for rats trained at ZT4. This behavior presents something of a paradox because it is the opposite of what the *c-Fos* data presents in both the IL and PL regions of the prefrontal cortex. Not every cell actively expresses *c-Fos*, making it a very interesting gene. *c-Fos* essentially has no basal expression but

is induced (*c-Fos* mRNA) minutes after there is a surge in activity above and beyond the regular neuronal firing rate (Cruz et al., 2015). Thus, *c-Fos* levels can show an important change in expression. When *c-Fos* is induced, it changes the neuroplasticity of the neuron, such that the neuron transitions into a state where it can engage in better neuroplasticity (Hogan et al., 2020). Thus, increased PL *c-Fos* expression in ZT16 rats tested at ZT4 (ZT16-36 h group) may reflect a prolonged need for these neurons to engage in neuroplasticity. This would align well with our behavioral data in suggesting that the ZT16 rats continue to consolidate the trace fear memory after ZT4 rats have already done so.

Taken together, these findings suggest that *Per1* and *c-Fos* showed similar expression patterns, with rats that recalled fear at ZT16 tending to have higher mRNA expression in the prefrontal cortex. While this experiment focused on trace fear recall without trace fear extinction, a future study to further understand trace fear learning and memory could be with the same basic design but with rats that are put through trace fear extinction learning and recall. Afterwards, the prefrontal cortex, specifically the IL and PL, could be analyzed for *Per1* and *c-Fos* mRNA expression to see how they differ from this experiment. We would hypothesize that *Per1* expression might look similar to what we have seen here, given the similarities we found across prefrontal cortex subdivisions, but we think that *c-Fos* expression patterns in the IL and PL might be reversed. Additionally, to determine if memory consolidation is responsible for the decrease in *Per1* for ZT4 rats, this experiment could be run again for only ZT4 trained rats with an additional ZT4-72 h group, and we would expect that this 72 h group would continue to show low levels of *Per1* expression.

Fear conditioning is a fundamental process in the study of learning and memory and is also relevant to our understanding of fear-based mental disorders. The results of this study are

significant because they reveal a previously unknown time-of-day difference in the recall of trace conditioned fear. Notably, this time-of-day difference does not parallel *Per1* or *c-Fos* mRNA expression in the prefrontal cortex. Clinically, understanding these emotional learning circuits could help to understand impairments in different forms of learning and memory in fear-based mental disorders. Studying trace fear conditioning can provide insights into the underlying mechanisms of these disorders and aid in the development of more effective assessments, treatments, and prevention, with a focus on circadian rhythms.

Acknowledgments

None of this could have been possible without the help of some very special people. I would first like to thank Dr. Robert Spencer for his guidance, expertise, and support ever since I joined the lab. Thank you for continually challenging me to learn and grow and for allowing me to defend an Honors Thesis, making it a fulfilling process. I would especially like to thank my graduate student mentor Dr. Matt Hartsock for being the best teacher, mentor, and role model during these undergraduate years. Thank you for helping me every step of the way with this honors defense and the hours you have spent teaching and guiding me, to you I owe my most gratitude. I would next like to thank my Honors Defense Counsel Dr. Lauren Chun and Dr. Jihye Park. Dr. Chun is a legend in the Spencer lab and her extensive research helped pave the way for this project. Dr. Park is a dear teacher to me, always pushing me to try my hardest and always there every step of the way. I would like to thank the Spencer lab, especially Will for helping me run some last minute *in situ's* to save my thesis. I could not have asked for better people to work with in a lab, thank you all. I would like to thank BSI for helping fund the hours poured into this project, and supporting me and other students with our research on campus. Thank you UROP for funding over the summer for this project. Last but not least, I want to thank my friends and family for helping me succeed, and listening to me talk about Neuroscience all these years.

References

Acheson, D. T., Eyler, L. T., Resovsky, J., Tsan, E., & Risbrough, V. B. (2015). Fear extinction memory performance in a sample of stable, euthymic patients with bipolar disorder. *Journal of Affective Disorders*, *185*, 230–238. https://doi.org/10.1016/j.jad.2015.06.053

Agorastos, A., & Olff, M. (2020). Traumatic stress and the circadian system: neurobiology, timing and treatment of posttraumatic chronodisruption. *European journal of psychotraumatology*, *11*(1), 1833644. https://doi.org/10.1080/20008198.2020.1833644

American Psychiatric Association. (2013). Cautionary statement for forensic use of DSM-5. In *Diagnostic and statistical manual of mental disorders* (5th ed.). https://doi.org/10.1176/appi.books.9780890425596.CautionaryStatement

Baltazar, R. M., Coolen, L. M., & Webb, I. C. (2013). Diurnal rhythms in neural activation in the mesolimbic reward system: Critical role of the medial prefrontal cortex. European Journal of Neuroscience, 38(2), 2319–2327. https://doi.org/10.1111/ejn.12224

Boivin DB, Boudreau P. (2014) Impacts of shift work on sleep and circadian rhythms. Pathology Biology. vol 62(5):292-301.

Bollinger T, Schibler U. (2014) Circadian rhythms - from genes to physiology and disease. Swiss Med Wkly. vol 24;144:w13984.

Bouton, M. E. (2000). A learning theory perspective on lapse, relapse, and the maintenance of behavior change. *Health Psychology*, *19*, 57–63. https://doi.org/10.1037/0278-6133.19.Suppl1.57

Bouton, M. E. (2002). Context, ambiguity, and unlearning: Sources of relapse after behavioral extinction. *Biological Psychiatry*, *52*(10), 976–986. https://doi.org/10.1016/s0006-3223(02)01546-9

Blum, S., Runyan, J. D., & Dash, P. K. (2006). Inhibition of prefrontal protein synthesis following recall does not disrupt memory for trace fear conditioning. *BMC Neuroscience*, *7*(1), 67. https://doi.org/10.1186/1471-2202-7-67

Chaudhury, D., & Colwell, C. S. (2002). Circadian modulation of learning and memory in fear-conditioned mice. Behavioural Brain Research, 133(1), 95–108. https://doi.org/10.1016/S0166-4328(01)00471-5

Chun, L. E., Woodruff, E. R., Morton, S., Hinds, L. R. & Spencer, R. L. Variations in Phase and Amplitude of Rhythmic Clock Gene Expression across Prefrontal Cortex, Hippocampus, Amygdala, and Hypothalamic Paraventricular and Suprachiasmatic Nuclei of Male and Female Rats. *Journal of biological rhythms* **30**, 417–436 (2015).

Chun, L. E. et al. Adrenal-dependent and -independent stress-induced Per1 mRNA in hypothalamic paraventricular nucleus and prefrontal cortex of male and female rats. Stress (Amsterdam, Netherlands) 21, 69–83 (2018).

Cirelli, C., Gutierrez, C. M., & Tononi, G. (2004). Extensive and divergent effects of sleep and wakefulness on brain gene expression. Neuron, 41(1), 35–43. https://doi.org/10.1016/S0896-6273(03)00814-6

Clark L, Cools R, & Robbins TW (2004, 6). The neuropsychology of ventral prefrontal cortex: decision-making and reversal learning. Brain Cogn, 55(1), 41–53. 10.1016/S0278-2626(03)00284-7

Conway-Campbell, B. L., Sarabdjitsingh, R. A., McKenna, M. A., Pooley, J. R., Kershaw, Y. M., Meijer, O. C., de Kloet, E. R., & Lightman, S. L. (2010). Glucocorticoid ultradian rhythmicity directs cyclical gene pulsing of the clock gene period 1 in rat hippocampus. *Journal of Neuroendocrinology*, *22*(10), 1093–1100.

https://doi.org/10.1111/j.1365-2826.2010.02051.x

Cox KH, Takahashi JS. (2019) Circadian clock genes and the transcriptional architecture of the clock mechanism. J Mol Endocrinol. vol 63(4):93-102

Craske et al., Nature Reviews "Anxiety Disorders" 2017

Cruz, F. C., Rubio, F. J., & Hope, B. T. (2015). Using c-fos to study neuronal ensembles in corticostriatal circuitry of addiction. *Brain Research*, *1628*(0 0), 157–173. https://doi.org/10.1016/j.brainres.2014.11.005

Daan S, & Pittendrigh CS (1976a). A functional analysis of circadian pacemakers in nocturnal rodents. *J Comp Physiol*, 106(3), 253–266. 10.1007/BF01417857

Daan S, & Pittendrigh CS (1976b). A functional analysis of circadian pacemakers in nocturnal rodents. *J Comp Physiol*, 106(3), 267–290. 10.1007/BF01417858

Davidson, J. R., Hughes, D., Blazer, D. G., & George, L. K. (1991). Post-traumatic stress disorder in the community: An epidemiological study. *Psychological Medicine*, *21*(3), 713–721. https://doi.org/10.1017/s0033291700022352

Davis, F. D., Bagozzi, R. P., & Warshaw, P. R. (1992). Extrinsic and Intrinsic Motivation to Use Computers in the Workplace1. *Journal of Applied Social Psychology*, *22*(14), 1111–1132. https://doi.org/10.1111/j.1559-1816.1992.tb00945.x

Dayan, J., Rauchs, G., & Guillery-Girard, B. (2016). Rhythms dysregulation: A new perspective for understanding PTSD? *Journal of Physiology, Paris*, *110*(4 Pt B), 453–460. https://doi.org/10.1016/j.jphysparis.2017.01.004

Duvarci, S., and Pare, D. (2014). Amygdala microcircuits controlling learned fear. *Neuron* 82, 966–980. doi: 10.1016/j.neuron.2014.04.042

Eckel-Mahan K, Sassone-Corsi P. (2013) Metabolism and the circadian clock converge. Physiol Rev. vol 93(1):107-35.

Evans, J. A., & Davidson, A. J. (2013). Health consequences of circadian disruption in humans and animal models. *Progress in Molecular Biology and Translational Science*, *119*, 283–323. https://doi.org/10.1016/B978-0-12-396971-2.00010-5

Facts & Statistics | Anxiety and Depression Association of America, ADAA. (2021, April 1).Anxiety & Depression Association of America. https://adaa.org/understanding-anxiety/facts-statistics

Fausnaught, A. B. (2021). *Examining time-of-day differences in the learning and recall of trace conditioned fear and trace conditioned fear extinction in male rats*. (Undergraduate thesis, University of Colorado Boulder) https://scholar.colorado.edu/concern/undergraduate_honors_theses/1n79h5440

Gang, E. (2018, September 26). Understanding the Brain of a PTSD Veteran. Veterans Disability Info. https://www.veteransdisabilityinfo.com/blog.php?article=understanding-the-brain-of-a-ptsd-veteran_374

Gilmartin, M. R., Balderston, N. L., & Helmstetter, F. J. (2014). Prefrontal cortical regulation of fear learning. *Trends in Neurosciences*, *37*(8), 455–464. https://doi.org/10.1016/j.tins.2014.05.004

Giustino, T. F., & Maren, S. (2015). The Role of the Medial Prefrontal Cortex in the Conditioning and Extinction of Fear. *Frontiers in Behavioral Neuroscience*, *9*. https://doi.org/10.3389/fnbeh.2015.00298

Gonzalez, S. T., & Fanselow, M. S. (2020). The role of the ventromedial prefrontal cortex and context in regulating fear learning and extinction. *Psychology & Neuroscience*, *13*, 459–472. https://doi.org/10.1037/pne0000207

Goode, T. D., & Maren, S. (2014). Animal models of fear relapse. *ILAR Journal*, *55*(2), 246–258. https://doi.org/10.1093/ilar/ilu008

Güler, A. D., Ecker, J. L., Lall, G. S., Haq, S., Altimus, C. M., Liao, H.-W., Barnard, A. R., Cahill, H., Badea, T. C., Zhao, H., Hankins, M. W., Berson, D. M., Lucas, R. J., Yau, K.-W., & Hattar, S. (2008). Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. *Nature*, *453*(7191), 102–105. https://doi.org/10.1038/nature06829

Haddon JE, & Killcross S (2007, 5). Contextual control of choice performance: behavioral, neurobiological, and neurochemical influences. Ann N Y Acad Sci, 1104, 250–269. 10.1196/annals.1390.000

Hartsock MJ, Spencer RL. (2020) Memory and the circadian system: Identifying candidate mechanisms by which local clocks in the brain may regulate synaptic plasticity. Neurosci Biobehav Rev. vol 118:134-162.

Hartsock, M. J., Strnad, H. K., & Spencer, R. L. (2022). Iterative Metaplasticity Across Timescales: How Circadian, Ultradian, and Infradian Rhythms Modulate Memory Mechanisms. *Journal of Biological Rhythms*, *37*(1), 29–42. https://doi.org/10.1177/07487304211058256

Hastings, M. H., Reddy, A. B., & Maywood, E. S. (2003). A clockwork web: Circadian timing in brain and periphery, in health and disease. *Nature Reviews Neuroscience*, *4*(8), 649–661. https://doi.org/10.1038/nrn1177

Hattar, S., Liao, H. W., Takao, M., Berson, D. M., & Yau, K. W. (2002). Melanopsin-containing retinal ganglion cells: Architecture, projections, and intrinsic photosensitivity. *Science (New York, N.Y.)*, *295*(5557), 1065–1070. https://doi.org/10.1126/science.1069609

Hermans, D., Craske, M. G., Mineka, S., & Lovibond, P. F. (2006). Extinction in human fear conditioning. *Biological Psychiatry*, *60*(4), 361–368. https://doi.org/10.1016/j.biopsych.2005.10.006

Hogan, M. K., Hamilton, G. F., & Horner, P. J. (2020). Neural Stimulation and Molecular Mechanisms of Plasticity and Regeneration: A Review. *Frontiers in Cellular Neuroscience*, *14*, 271. https://doi.org/10.3389/fncel.2020.00271

Jones, J. R., Chaturvedi, S., Granados-Fuentes, D., & Herzog, E. D. (2021). Circadian neurons in the paraventricular nucleus entrain and sustain daily rhythms in glucocorticoids. *Nature Communications*, *12*(1), Article 1. https://doi.org/10.1038/s41467-021-25959-9

Kesner RP, & Churchwell JC (2011, 10). An analysis of rat prefrontal cortex in mediating executive function. Neurobiol Learn Mem, 96(3), 417–431. 10.1016/j.nlm.2011.07.002

Kesner RP, Hunt ME, Williams JM, & Long JM (1996, 1996 Mar-Apr). Prefrontal cortex and working memory for spatial response, spatial location, and visual object information in the rat. Cereb Cortex, 6(2), 311–318. 10.1093/cercor/6.2.311

Kim, J. J., and Fanselow, M. S. (1992). Modality-specific retrograde amnesia of fear. *Science* 256, 675–677. doi: 10.1126/science.1585183

Kim, J., Lee, S., Park, H., Song, B., Hong, I., Geum, D., et al., (2007). Blockade of amygdala metabotropic glutamate receptor subtype 1 impairs fear extinction. *Biochem. Biophys. Res. Commun.* 355, 188–193. doi: 10.1016/j.bbrc.2007.01.125

Knutsson, A. (2003). Health disorders of shift workers. *Occupational Medicine (Oxford, England)*, *53*(2), 103–108. https://doi.org/10.1093/occmed/kqg048

Kovács, K. J. (2008). Measurement of immediate-early gene activation- c-fos and beyond. *Journal of Neuroendocrinology*, *20*(6), 665–672.

https://doi.org/10.1111/j.1365-2826.2008.01734.x

Krishnan, H. C., & Lyons, L. C. (2015). Synchrony and desynchrony in circadian clocks: Impacts on learning and memory. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *22*(9), 426–437. https://doi.org/10.1101/lm.038877.115

Kwapis JL, Jarome TJ, Schiff JC, Helmstetter FJ. Memory consolidation in both trace and delay fear conditioning is disrupted by intra-amygdala infusion of the protein synthesis inhibitor anisomycin. Learn Mem. 2011 Oct 25;18(11):728-32. doi: 10.1101/lm.023945.111. PMID: 22028394; PMCID: PMC3207254.

Kwapis, J. L., Jarome, T. J., & Helmstetter, F. J. (2014). The role of the medial prefrontal cortex in trace fear extinction. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *22*(1), 39–46. https://doi.org/10.1101/lm.036517.114

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annual Review of Neuroscience*, *23*, 155–184. https://doi.org/10.1146/annurev.neuro.23.1.155

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annual Review of Neuroscience*, *24*, 897–931. https://doi.org/10.1146/annurev.neuro.24.1.897

Maren, S. (2014). Nature and causes of the immediate extinction deficit: A brief review. *Neurobiology of Learning and Memory*, *113*, 19–24. https://doi.org/10.1016/j.nlm.2013.10.012

Maren, S., Phan, K. L., & Liberzon, I. (2013). The contextual brain: Implications for fear conditioning, extinction and psychopathology. *Nature Reviews. Neuroscience*, *14*(6), 417–428. https://doi.org/10.1038/nrn3492

Maren, S., & Chang, C. (2006). Recent fear is resistant to extinction. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(47), 18020–18025.

https://doi.org/10.1073/pnas.0608398103

Martin, E. I., Ressler, K. J., Binder, E., & Nemeroff, C. B. (2009). The neurobiology of anxiety disorders: brain imaging, genetics, and psychoneuroendocrinology. *The Psychiatric clinics of North America*, *32*(3), 549–575. https://doi.org/10.1016/j.psc.2009.05.004

McDonald, A. J., and Augustine, J. R. (1993). Localization of GABA-like immunoreactivity in the monkey amygdala. *Neuroscience* 52, 281–294. doi: 10.1016/0306-4522(93)90156-a

Mental disorders. (2022.). Retrieved December 6, 2022, from https://www.who.int/news-room/fact-sheets/detail/mental-disorders

Mifsud, K. R., & Reul, J. M. H. M. (2016). Acute stress enhances heterodimerization and binding of corticosteroid receptors at glucocorticoid target genes in the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(40), 11336–11341. https://doi.org/10.1073/pnas.1605246113

Milad, M. R., Pitman, R. K., Ellis, C. B., Gold, A. L., Shin, L. M., Lasko, N. B., Zeidan, M. A., Handwerger, K., Orr, S. P., & Rauch, S. L. (2009). Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biological Psychiatry*, *66*(12), 1075–1082. https://doi.org/10.1016/j.biopsych.2009.06.026

Milad, M. R., and Quirk, G. J. (2012). Fear extinction as a model for translational neuroscience: ten years of progress. *Annu. Rev. Psychol.* 63, 129–151. doi: 10.1146/annurev.psych.121208.131631

Moore, R. Y., & Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research*, *42*(1), 201–206. https://doi.org/10.1016/0006-8993(72)90054-6

Myers, K. M., & Davis, M. (2007). Mechanisms of fear extinction. *Molecular Psychiatry*, *12*(2),

120–150. https://doi.org/10.1038/sj.mp.4001939

National Institute of Mental Health. (2022). National Institute of Mental Health (NIMH). https://www.nimh.nih.gov/health/statistics/any-anxiety-disorder

Nitecka, L., and Ben-Ari, Y. (1987). Distribution of GABA-like immunoreactivity in the rat amygdaloid complex. *J. Comp. Neurol.* 266, 45–55. doi: 10.1002/cne.902660105

Norrholm, S. D., Jovanovic, T., Olin, I. W., Sands, L. A., Karapanou, I., Bradley, B., & Ressler, K. J. (2011). Fear extinction in traumatized civilians with posttraumatic stress disorder: Relation to symptom severity. *Biological Psychiatry*, *69*(6), 556–563. https://doi.org/10.1016/j.biopsych.2010.09.013

Orr, S. P., Metzger, L. J., Lasko, N. B., Macklin, M. L., Peri, T., & Pitman, R. K. (2000). De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. *Journal of Abnormal Psychology*, *109*(2), 290–298.

Orsini, C. A., & Maren, S. (2012). Neural and cellular mechanisms of fear and extinction memory formation. *Neuroscience & Biobehavioral Reviews*, *36*(7), 1773–1802. https://doi.org/10.1016/j.neubiorev.2011.12.014

Pace-Schott, E. F., Spencer, R. M. C., Vijayakumar, S., Ahmed, N., Verga, P. W., Orr, S. P., Pitman, R. K., & Milad, M. R. (2013). Extinction of Conditioned Fear is Better Learned and Recalled in the Morning than in the Evening. *Journal of Psychiatric Research*, *47*(11), 1776–1784. https://doi.org/10.1016/j.jpsychires.2013.07.027

Pace-Schott, E. F., Germain, A., & Milad, M. R. (2015). Effects of sleep on memory for conditioned fear and fear extinction. *Psychological bulletin*, *141*(4), 835–857. https://doi.org/10.1037/bul0000014

Paré, D., and Smith, Y. (1993). Distribution of GABA immunoreactivity in the amygdaloid

complex of the cat. Neuroscience 57, 1061–1076. doi: 10.1016/0306-4522(93)90049-l

Patke A, Young MW, Axelrod S. (2020) Molecular mechanisms and physiological importance of circadian rhythms. Nat Rev Mol Cell Biol. vol 21(2):67-84. Pittendrigh CS, & Daan S (1976a). A functional analysis of circadian pacemakers in nocturnal rodents. *J Comp Physiol*, 106(3), 223–252. 10.1007/BF01417856

Pavlov, I. P. (1927). Conditioned reflexes: An investigation of the physiological activity of the cerebral cortex (G. von Anrep, Trans.). Oxford University Press

Phillips, R. G., and Ledoux, J. E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* 106, 274–285. doi: 10.1037/0735-7044.106.2.274

Pittendrigh CS, & Daan S (1976a). A functional analysis of circadian pacemakers in nocturnal rodents. *J Comp Physiol*, 106(3), 223–252. 10.1007/BF01417856

Pittendrigh CS, & Daan S (1976b). A functional analysis of circadian pacemakers in nocturnal rodents. *J Comp Physiol*, 106(3), 291–331. 10.1007/BF01417859

Pittendrigh CS, & Daan S (1976c). A functional analysis of circadian pacemakers in nocturnal rodents. *J Comp Physiol*, 106(3), 333–355. 10.1007/BF01417860

Pittendrigh CS, & Minis DH (1964). The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *Am Nat*, 98(902), 261–294. 10.1086/282327

Post-Traumatic Stress Disorder (PTSD). (2022). National Institute of Mental Health (NIMH). Retrieved December 9, 2022, from https://www.nimh.nih.gov/health/statistics/post-traumatic-stress-disorder-ptsd

Quirk, G. J., and Beer, J. S. (2006). Prefrontal involvement in the regulation of emotion: convergence of rat and human studies. *Curr. Opin. Neurobiol.* 16, 723–727. doi:

10.1016/j.conb.2006.07.004

Radulovic, J., Kammermeier, J., & Spiess, J. (1998). Relationship between Fos Production and Classical Fear Conditioning: Effects of Novelty, Latent Inhibition, and Unconditioned Stimulus Preexposure. *The Journal of Neuroscience*, *18*(18), 7452–7461. https://doi.org/10.1523/JNEUROSCI.18-18-07452.1998

Ralph, M. R., Foster, R. G., Davis, F. C., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science (New York, N.Y.)*, *247*(4945), 975–978. https://doi.org/10.1126/science.2305266

Rawashdeh, O., Parsons, R., & Maronde, E. (2018). Clocking in time to gate memory processes: The circadian clock is part of the ins and outs of memory. *Neural Plasticity*, *2018*. https://doi.org/10.1155/2018/6238989

Rehman, I., Mahabadi, N., Sanvictores, T., & Rehman, C. I. (2022). Classical Conditioning. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK470326/

Reppert, S. M., & Weaver, D. R. (2001). Molecular analysis of mammalian circadian rhythms. *Annual Review of Physiology*, *63*, 647–676. https://doi.org/10.1146/annurev.physiol.63.1.647

Riga, D., Matos, M. R., Glas, A., Smit, A. B., Spijker, S., and Van den Oever, M. C. (2014). Optogenetic dissection of medial prefrontal cortex circuitry. *Front. Syst. Neurosci.* 8:230. doi: 10.3389/fnsys.2014.00230

Rosellini, A. J., Liu, H., Petukhova, M. V., Sampson, N. A., Aguilar-Gaxiola, S., Alonso, J., Borges, G., Bruffaerts, R., Bromet, E. J., de Girolamo, G., de Jonge, P., Fayyad, J., Florescu, S., Gureje, O., Haro, J. M., Hinkov, H., Karam, E. G., Kawakami, N., Koenen, K. C., … Kessler, R. C. (2018). Recovery from DSM-IV post-traumatic stress disorder in

the WHO World Mental Health surveys. *Psychological Medicine*, *48*(3), 437–450. https://doi.org/10.1017/S0033291717001817

Royer, S., Martina, M., and Paré, D. (1999). An inhibitory interface gates impulse traffic between the input and output stations of the amygdala. *J. Neurosci.* 19, 10575–10583.

Saini, C., Brown, S. A., & Dibner, C. (2015). Human Peripheral Clocks: Applications for Studying Circadian Phenotypes in Physiology and Pathophysiology. *Frontiers in Neurology*, *6*, 95. https://doi.org/10.3389/fneur.2015.00095

Saper, C. B. (2013). The Central Circadian Timing System. *Current Opinion in Neurobiology*, *23*(5), 747–751. https://doi.org/10.1016/j.conb.2013.04.004

Selden, N. R. W., Everitt, B. J., Jarrard, L. E., and Robbins, T. W. (1991). Complementary roles for the amygdala and hippocampus in aversive conditioning to explicit and contextual cues. *Neuroscience* 42, 335–350. doi: 10.1016/0306-4522(91)90379-3

So, A. Y.-L., Bernal, T. U., Pillsbury, M. L., Yamamoto, K. R., & Feldman, B. J. (2009). Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(41), 17582–17587. https://doi.org/10.1073/pnas.0909733106

Sotres-Bayon, F., and Quirk, G. J. (2010). Prefrontal control of fear: more than just extinction. *Curr. Opin. Neurobiol.* 20, 231–235. doi: 10.1016/j.conb.2010.02.005

Spencer, R. L., Chun, L. E., Hartsock, M. J., & Woodruff, E. R. (2018). Glucocorticoid hormones are both a major circadian signal and major stress signal: How this shared signal contributes to a dynamic relationship between the circadian and stress systems. *Frontiers in Neuroendocrinology*, *49*, 52–71. https://doi.org/10.1016/j.yfrne.2017.12.005

This is the world's biggest mental health problem—And you might not have heard of it. (2022).

World Economic Forum. Retrieved December 6, 2022, from https://www.weforum.org/agenda/2019/01/this-is-the-worlds-biggest-mental-health-problem/

Walker, W. H., Walton, J. C., DeVries, A. C., & Nelson, R. J. (2020). Circadian rhythm disruption and mental health. *Translational Psychiatry*, *10*(1), Article 1. https://doi.org/10.1038/s41398-020-0694-0

Woodruff, E. R., Greenwood, B. N., Chun, L. E., Fardi, S., Hinds, L. R., & Spencer, R. L. (2015). Adrenal-dependent diurnal modulation of conditioned fear extinction learning. *Behavioural Brain Research*, *286*, 249–255. https://doi.org/10.1016/j.bbr.2015.03.006

Woodruff, E. R., Chun, L. E., Hinds, L. R., Varra, N. M., Tirado, D., Morton, S. J., McClung, C. A., & Spencer, R. L. (2018). Coordination between Prefrontal Cortex Clock Gene Expression and Corticosterone Contributes to Enhanced Conditioned Fear Extinction Recall. Eneuro, 5(6), ENEURO.0455-18.2018. https://doi.org/10.1523/eneuro.0455-18.2018

Vervliet, B., Craske, M. G., & Hermans, D. (2013). Fear extinction and relapse: State of the art. *Annual Review of Clinical Psychology*, *9*, 215–248. https://doi.org/10.1146/annurev-clinpsy-050212-185542

Yamamoto, T., Nakahata, Y., Tanaka, M., Yoshida, M., Soma, H., Shinohara, K., Yasuda, A., Mamine, T., & Takumi, T. (2005). Acute physical stress elevates mouse Period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. Journal of Biological Chemistry, 280(51), 42036–42043. https://doi.org/10.1074/jbc.M509600200

Yoo, S.-H., Yamazaki, S., Lowrey, P. L., Shimomura, K., Ko, C. H., Buhr, E. D., Siepka, S. M.,

Hong, H.-K., Oh, W. J., Yoo, O. J., Menaker, M., & Takahashi, J. S. (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences*, *101*(15), 5339–5346. https://doi.org/10.1073/pnas.0308709101